Evaluation of chemicals for Toxic & Teratogenic Effects Using the Chick Embryo as the test system-FDA Contract #71-331 **(Sodium Metabisulfite)** FDA Compound #71-22

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FDA CONTACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

SODIUM METABISULFITE: FDA 71-22

WARF INSTITUTE, INC.
MADISON, WISCONSIN

FDA CONTRACT 71-331

EVALUATION OF CHEMICALS FOR TOXIC AND TERATOGENIC EFFECTS USING THE CHICK EMBRYO AS THE TEST SYSTEM

Objective: To determine the toxic and teratogenic effects of GRAS List compounds when injected into the air cell and yolk of fertile chicken eggs.

Procedure:

A. Test System and Incubation Procedures:

Fertile hatching eggs were chosen from a single comb white leghorn breeder flock. The eggs were candled and graded to eliminate internal and external defects; blood and meat spots, tremulous air cells, rough or cracked shells. The eggs chosen for injection weighed from 23 - 26 ounces/ dozen and were not washed or dipped. The eggs were gathered within 48 hours of the injection or incubation and were held at 50 - 60°F. and 60 - 80% relative humidity. The breeder ration fed the flock was formulated by the breeder to meet or exceed the recommendations of the Nutrient Requirements of Poultry, Number 1 - 1971, National Academy of Sciences, and contained no additions of antibiotics, arsenicals, nitrofurazones or similar chemical additives. The breeder flock was blood tested and negative for pullorum-typhoid and mycoplasm gallispecticum.

Eggs were incubated in Jamesway 1080 forced air incubators equipped with automatic controls to regulate temperature, humidity and egg turning. Temperature and relative humidity were maintained at 99.5°F. and 86°F.* wet bulb respectively for the first 18 days of incubation and eggs were turned each two hours. The eggs were then transfered to the hatcher in 3½" x 5" x 25" covered hardware cloth hatching baskets for the hatching period. Temperature in

* 86°F. wet bulb refers to temperature of wet bulb apparatus in standard incubator hatching equipment and is equivalent to approximately 56% relative humidity.

the hatcher was maintained at 98.5oF. and relative humidity at 86oF. wet bulb. When the humidity had risen to 88 degrees as a result of moisture generated by hatching the hatcher was adjusted to hold 88oF. wet bulb relative humidity until the chicks were removed on the morning of the 23rd day of incubation.

Prior to incubating or hatching each setting of eggs and following each hatching the incubator and its metal parts were thoroughly cleaned by vacuuming and washing with a 200 ppm solution of "ROCCAL" which contains 10% alkyl (C12, C14, C16 and related alkyl groups from C8 to C18) - dimethyl benzyl ammonium chloride. The sanitized surfaces were allowed to completely air dry prior to the introduction of eggs. Following each transfer of eggs to the hatching compartment, and when temperature and humidity had returned to normal levels, the hatcher and the eggs it contained were fumigated by combining 10 grams of potassium permanganate crystals and 20 grams of 37% formaldahyde solution.

B. Test Sample Preparation and Administration:

The test sample was taken up in an appropriate solvent to facilitate administration at the levels chosen. Sterile glassware, syringes and needles were employed to prepare and administer the test sample or solvent. Eggs previously selected were candled and the location of the air cell marked with pencil. The eggs were then randomized into the experimental groups. Injection of solvent or test sample dilution was accomplished by placing the material on the air cell membrane or by injection into the yolk sac. These administrations were made at both 0 and 96 hours of incubation. For the 0 hour experiments the eggs were assumed to be fertile; however, in the 96 hour experiments the eggs were candled as previously described and only those eggs with a well developed 96 hour embryo were selected for use.

1. Air Cell Administration:

The eggs were wiped at the injection site with 70% ethanol and allowed to air dry. A hole measuring approximately 6mm was then drilled over the air cell in each egg using a "Dremel Moto-tool", model 270. The cutter employed deflected the shell fragments upwards and outwards. Remaining shell membrane fragments were removed with a small

forceps and the surface of the egg membrane visually examined for damage. The solvent or test sample was then deposited on the egg membrane with a model SB2 Syringe Microburet. Immediately following, the hole was sealed with ½" Scotch Brand transparent tape. Two additional groups of eggs were normally included with each air cell experiment; a group which had been drilled, shell membrane fragments removed, and sealed only and a group of control eggs which had received no treatments whatsoever.

2. Yolk Administration:

The eggs were placed within a Fisher Scientific "Isolator/Lab" equipped with plastic irises through which the hands and forearms were placed during injection. Prior to injection the eggs and miscellaneous required equipment were submitted to a fumigation of 1.8 grams of potassium permanganate crystals and 3.6 grams of 37% formaldahyde. The eggs were held in this atmosphere for 30 minutes prior to further handling.

Each egg was then wiped at the injection site with 70% ethanol and allowed to air dry. A small hole was engraved directly over the air cell with a Burgess model V-13 Vibro-Graver. Care was taken not to damage the membrane attached to the shell. The surface of the egg at the engraved site was vacuumed to remove the shell particles produced. The egg was then slid onto the needle of the Syringe Microburet with the egg horizontal on its long axis until the top of the egg reached the hub of the 1" - 25 ga. hypodermic needle. Following the injection of the material into the yolk sac, the egg was carefully withdrawn from the needle and the hole sealed with transparent tape. The hypodermic needle was carefully wiped with a sterile gauze pad prior to the next injection. As in the air cell administration, normally two additional groups of eggs were included in each yolk experiment; a group which had been drilled, pierced with the hypodermic needle and sealed only and a group of control eggs which had received no treatment other than fumigation.

Following the air cell and yolk injections the eggs were identified as to experiment and group with a No. 3 lead pencil and were then incubated as described above.

C. Test Profile:

The work was divided into one or more Preliminary Range Finding Experiments, two Dose-Response and Teratogenic Experiments, and Ancillary Investigations (Post Hatch Trials).

1. Preliminary Range Finding Experiments:

The objective of these trials was to locate the approximate LD-50 of the test sample. This data was used to design the dose levels for the Dose-Response trials. The test sample and solvent were administered by two routes; air cell and yolk, and at 0 and 96 hours of incubation. In general, at each route and time of incubation, 5 volumes of test sample dilution were administered together with 5 levels of solvent at the same volumes. Control eggs were also usually included as described above. Normally 10-20 eggs were used per group in these trials. When necessary these trials were repeated in an effort to locate the approximate LD-50 for the test compound.

Beginning on the 6th day of the incubation, the eggs set in the Preliminary Range Finding Experiments were candled daily and non-viable embryos removed. These embryos were examined grossly for determination of developmental age and evidence of teratogenic effect, however, mortality was the main parameter in these trials. The remaining eggs were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The resultant chicks and non-viable embryos were examined grossly for teratogenic effects and all pertinent data was recorded. An estimate of the LD-50 for the test compound was then made.

2. Dose-Response and Teratogenic Experiments:

Based upon information from the Preliminary Range-Finding Experiments, the Dose-Response Experiment was designed, employing 5 levels of sample dilution expected to produce mortality from the background level up through approximately 90%. Five volume levels of solvent were included as solvent controls at each route and time of administration. Normally 10 eggs/group were used in the solvent series with 50 eggs/group for the test sample dilutions. Twenty eggs/group were normally included for the drilled or pierced and non-treated controls. Two such experiments were conducted for each sample so that ultimately 100 eggs were tested on each test dilution at each route and stage of incubation.

The eggs set in these experiments were candled daily beginning on the 6th day of incubation and the non-viable embryos removed for examination as previously described. Where necessary, embryos were examined with the aid of a dissecting microscope. Remaining embryos were transferred to the hatching compartment on the 18th day of incubation and allowed to hatch. The apparently normal chicks were then removed from the hatching trays and examined externally for anomalies.

Remaining non-viable embryos and chicks which were alive but unable to hatch were individually examined externally for abnormalities. These non-viable embryos, chicks which were alive but unable to hatch, and a portion of the normal chicks were examined in one aspect by X-ray. The chicks and embryos which had been X-rayed and all remaining normal chicks were then examined internally for possible anomalies of the viscera. All pertinent data were recorded.

3. Post Hatch Trials:

Apparently normal chicks were chosen from one 50 egg experiment for this portion of the study.

Generally 20 chicks (straight-run) were wing banded from each level chosen and were placed in Jamesway electrically heated battery brooders. Central Soya Chick Starter was fed as the sole ration to 8 weeks of age and Central Soya Grower from 8 weeks of age to termination. These diets were non-medicated. The chicks chosen were usually from the approximate LD-50 and no-effect levels for the test compound from each route of administration and time of incubation. Negative control, untreated chicks, were also included. In some cases chicks were chosen from groups where a relatively high incidence of anomalies were seen rather than from the LD-50 or no-effect levels specifically. Body weight data were collected weekly through 4 weeks of age and bi-weekly to termination. Average group feed consumption was recorded periodically.

4. Histopathology:

A random sampling of birds from selected groups were specified for histologic examination. These chicks comprised 5 males and 5 females from the test groups selected and 5 males and 5 females from a negative control group. Groups to be sampled were selected on the basis of observations of specific effects and a judgment made as to what groups would give the most information from the limited histopathologic examination.

The chicks sacrificed were either day old or varying ages in a Post Hatch Trial. The following tissues were collected, trimmed, dehydrated, embedded in paraffin, sectioned and stained with hematoxilin and eosin:

1. Thyroid
2. Liver
3. Spleen
4. Pancreas
5. Lung
6. Heart
7. Kidney
8. Gonad
9. Bursa

The prepared slides were examined and remarkable alterations noted.

Results:

The data developed in the testing of Sodium Metabisulfite are presented in the following tables:

Sodium Metabisulfite

Table 1 - Air Cell At 0 Hours
Table 2 - Air Cell At 96 Hours
Table 3 - Yolk At 0 Hours
Table 4 - Yolk At 96 Hours
Table 5 - Body Weight Data - Straight Run
Table 6 - Histopathology - Grow-Out Birds

In Tables 1 through 4, the following comments apply:

Column 1 gives the dose administered milligrams per kilogram, respectively. (The milligrams per kilogram figure is based on an average egg weight of fifty grams).

Column 2 is the total number of eggs treated.

Column 3 is the percent mortality, i.e., total non-viable divided by total treated eggs.

Column 4 is the total number of abnormal birds expressed as a percentage of the total eggs treated. This includes all abnormalities observed and also toxic response such as edema, hemorrhage, hypopigmentation of the down and other disorders such as feather abnormalities, significant growth retardation, cachexia, ataxia or other nerve disorders.

Column 5 is the total number of birds having a structural abnormality of the head, viscera, limb, or body skeleton expressed as percentage of the total eggs treated. Toxic response and disorders such as those noted for column 4 are not included.

Column 2 through 5 have been corrected for accidental deaths if any occurred. Included in these columns are comparable data for the solvent-treated eggs and the untreated controls.

The mortality data in column 3 have been examined for a linear relationship between the probit percent mortality versus the logarithm of the dose. The results are indicated at the bottom of each table.

The data of columns 3, 4 and 5 have been analyzed using the Chi Square test for significant differences from the solvent background. Each dose level is compared to the solvent value and levels of percent mortality or percent abnormalities that are significant (probability of being the same is 5% or less) are indicated by an asterisk in the tables. All values so indicated have a higher incidence than the solvent values.

Discussion:

The comments and data which follow concern the results obtained when Sodium Metabisulfite was employed in the test system.

Significant mortality (P .05) was seen in the 0 hour air cell treatments at levels of 7.3, 18.3, 36.7, 55.0 and 110.0 mg/kg when compared with solvent treated controls. The calculated LD-50 was 19.49 mg/kg. At 96 hour air cell, the mortality was significantly elevated at dose levels of 3.7, 5.5, 9.2 and 18.3 mg/kg, with a calculated LD-50 of 3.35 mg/kg.

In the 0 hour yolk treatments, mortality was significantly elevated at the upper five dose levels; 7.3, 18.3, 36.7, 55.0 and 110.0 mg/kg. The LD-50 was 53.04 mg/kg. In the 96 hour yolk treatments, the percent mortality was significantly elevated at dose levels of 73.3, 110.0, 220.0, 330.0 and 440.0 mg/kg, with an LD-50 of 161.49 mg/kg.

Significantly elevated levels of abnormalities were observed in the air cell at 0 hours and the yolk at 96 hours.

In the 0 hour air cell, the levels of total abnormalities were significantly elevated only at 36.7 and 110.0 mg/kg. Dwarfism or retarded development was the major contributor to the total percent abnormalities at these dose levels. In addition, at 36.7 mg/kg in the 98 eggs tested, several abnormalities of the limbs were observed which had not been seen in the flock background of 3315 eggs or in the control eggs for this time and route. Included were a single occurrence each of phocomelia, exostosis,micromelia and short toes. In addition, 2 occurrences of oligodactly were encountered. In the total of approximately 500 eggs tested at the upper 5 dose levels, 5 instances of flexed mandible and 3 parrot beaks were observed. These anomalies were not seen in solvent or untreated control eggs for this time and route, but occur in the flock background at 0.06 percent. The flock background is a total of all the drilled, pierced and untreated control eggs which were used in all the 50 egg studies using eggs from breeder flock N-1.

In 96 hour air cell treatments, the mortality at the upper 3 dose levels was 95% or greater with 75% of the deaths occurring prior to day 5. At the lower 3 dose levels, where mortality was decreased, two interesting embryos were seen in the 0.9 mg/kg dose level. The first, a 22 day embryo, with exencephaly, nevus (yolk sack tissue joined to tissue covering the brain) and congenital malposition and the second, a 4 day embryo with severe buphthalmia of the right eye. Neither nevus or buphthalmia had been seen in control eggs for this time or route or in the flock background. Several other low incidence anomalies were seen which had occurred previously in the flock background.

While percent total abnormalities were not significantly elevated at 0 hour yolk, it was noted that in the approximately 500 eggs tested at this time and route, ten anomalies of the head were observed. Included were flexed mandible, parrot beak, malformed maxilla, short mandible, cleft palate and exencephaly. With with the exception of exencephaly, these anomalies were not seen in the control eggs for this time and route but had been observed in the flock background. (Flexed mandible and parrot beak occur in the flock background at 0.06%, short mandible at 0.09%, exencephaly at 0.09% and cleft palate at 0.03%).

In the 96 hour yolk treatments, percent total abnormalities were significantly elevated at the upper 3 dose levels; 220.0, 330.0 and 440.0 mg/kg. Dwarfism, or retarded development, was the primary observation; however, important abnormalities of the head and a toxic response (edema) were also seen. Approximately 250 eggs were in-

volved and the anomalies mentioned above were seen at the following frequencies; flexed mandible (11), parrot beak (8) and generalized edema (14). These anomalies were not seen in control eggs for this time and route, but appear in the flock background at low levels, parrot beak and flexed mandible (.06%) and edema (.09%).

Dwarfism, retarded development, was a frequent observation in this experiment. To clarify our designation of dwarfism we will explain our classification procedure. At the first candling, day 5 or 6, we did not classify any embryos as retarded unless they were alive and definitely younger in development than 5 or 6 days. In subsequent candling any dead embryo judged by size to be 3 days behind in development was labeled as slight dwarfism, 4 days behind was labeled moderate dwarfism and 5 days or more behind was labeled severe dwarfism. If embryos were removed alive, 1 day behind was labeled slight, 2 days behind was labeled moderate and 3 days behind was labeled severe dwarfism. At hatch time an 18 day embryo was classified slightly dwarfed, a 17 day embryo was classified as moderate dwarfism and a 16 day embryo was classified as severe dwarfism. One might suspect that at the toxic levels administered, embryo development could be delayed due to metabolic or nutritional alterations that produced temporary growth depression which would not result in a permanent growth defect. Chicks which hatched were of normal size and no evidence of permanent growth retardation was observed.

Chicks from various control and treatment groups were raised to 4 weeks of age as indicated in the attached tables. Body weight gains and feed consumption were considered normal for all groups.

Five males and 5 females from the 0 hour air cell, 7.3 and 55.0 mg/kg dose, were sacrificed at approximately 4 weeks of age and examined grossly. No gross abnormalities were observed. Tissues selected from each bird were examined histologically. These tissues were compared with those from similar age untreated controls. The alterations noted were minimal in nature and randomly distributed among the various groups with the exception of an interstitial nephritis seen in kidney tissue of birds which received 7.3 mg/kg of the test material. This lesion was seen in the kidney of 8 of 10 birds examined.

X-ray examination did not reveal any abnormalities not already noted during daily examination of embryos.

Conclusion:

Under the conditions specified for this trial, Sodium Metabisulfite was particularly toxic when administered in the air cell at 0 and 96 hours and produced significantly elevated levels of abnormalities in the air cell treatments at 0 hours and in yolk treatments at 96 hours. While temporary growth retardation during in-

cubation was observed to be the major contributor to total abnormalities, a low level of structural anomalies involving the head and/or limbs was encountered which may suggest that further study with this compound is warranted.

Signed _____

By and For WARF Institute, Inc.

November 11, 1974

Test Sample: Sodium Metabisulfite

Identification: FDA 71-22

Solvent System: 10% Absolute Ethanol in sterile distilled H_2O

Breeder Flock: N-1

Preliminary Range Finding Experiments

Experiment No.	Initiated
30	5-30-72

Dose Response Experiments

Experiment No.	Initiated
34	6-26-72
47	9-18-72

Table 1

Sodium Metabisulfite
Air Cell At 0 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
110.0	109	95.41*	40.36*	2.75
55.0	105	81.90*	25.71	9.52
36.7	98	80.61*	31.63*	6.12
18.3	108	43.51*	16.66	1.85
7.3	109	29.35*	13.76	3.66
3.7	10	20.00	.00	.00
10% Ethanol	98	11.22	16.32	6.12
Drilled Control	50	6.00	6.00	2.00
Control/ Control	164	6.70	4.26	1.82

** LD-50 19.49 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 2

Sodium Metabisulfite
Air Cell At 96 Hours

Dose Mg/Kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
18.3	60	95.00*	.00	.00
9.2	109	97.24*	.00	.00
5.5	50	96.00*	.00	.00
3.7	109	55.04*	5.50	.91
1.8	108	14.81	4.62	3.70
.92	109	19.26	10.09	4.58
10% Ethanol	98	10.20	7.14	5.10
Drilled Control	40	5.00	5.00	2.50
Control/ Control	164	6.70	4.26	1.82

** LD-50 3.35 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 3
Sodium Metabisulfite
Yolk At 0 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
110.0	109	75.22*	17.43	2.75
55.0	107	63.55*	19.62	1.86
36.7	59	52.54*	23.72	5.08
18.3	110	60.00*	9.09	2.72
7.3	108	45.37*	12.96	.92
1.8	49	40.81	8.16	4.08
10% Ethanol	110	30.90	11.81	3.63
Pierced Control	50	20.00	6.00	2.00
Control/ Control	164	6.70	4.26	1.82

** LD-50 53.04 mg/kg

*Significantly different from solvent ($P \leq .05$)

Table 4
Sodium Metabisulfite
Yolk At 96 Hours

Dose mg/kg	Number Of Eggs	Percent** Mortality	Percent Abnormal	
			Total	Structural
440.0	98	96.93*	56.12*	7.14
330.0	48	93.75*	50.00*	10.41
220.0	99	58.58*	30.30*	5.05
110.0	110	35.45*	6.36	.90
73.3	99	25.25*	10.10	3.03
55.0	10	10.00	.00	.00
36.7	58	5.17	3.44	.00
18.3	9	11.11	11.11	.00
7.3	10	.00	10.00	.00
10% Ethanol	90	12.22	11.11	5.55
Pierced Control	50	6.00	8.00	.00
Control/ Control	164	6.70	4.26	1.82

** LD-50 161.49 mg/kg

* Significantly different from solvent ($P \leq .05$)

Table 5
Body Weight Data - Post Hatch Response
FDA 71-22: Sodium Metabisulfite
(Straight Run)

Test Dose (1)	Time/Route	Average Individual Body Weight - Grams Week 1	2	3	4
-	Control/Control	61	108	182	273
55.0	O/AC	60	107	177	265
7.3	O/AC	67	110	194	279
55.0	O/Y	59	103	176	252
1.8	O/Y	64	109	192	282
3.7	96/A	56	99	184	276
0.9	96/A	56	100	173	266
220.0	96/Y	58	105	183	274
36.7	96/Y	59	105	184	275

(1) Milligrams/Kilogram Of Body Weight

Table 6
Sodium Metabisulfite
Histopathology - Grow-Out Birds

Histologic Observations	Negative Control (10)	O/AC 55 MG/KG (10)	O/AC 7.3 MG/KG (10)
Thyroid			
increased cellularity		3	1
focal areas of cellular inflitration	1		1
focal areas of leukocytic infiltration			3
area of cellular infiltration under capsule	1		
less colloid		1	4
Lungs			
congested	3		
Heart			
focal areas of cellular infiltration	1		
Spleen			
peripheral pigmentation	8	1	
diffuse mild/moderate pigmentation		8	10
Liver			
focal area of pigmentation			1
diffuse pigmentation	1		3
peripheral pigmentation	6		
focal areas of cellular infiltration	9	4	2
focal area of leukocytic infiltration		1	3
Proventriculus			
focal areas of cellular infiltration	1		
Small Intestine			
focal lymphatic infiltration		1	
Pancreas			
degenerate	1		
Gonads			
testicles immature	1		
testicles degenerate	1		
Kidney			
minimal interstitial nephritis (focal area of cellular infiltration)		2	8
atrophy of glomeruli (mild or moderate)		2	
focal area of degeneration		1	1